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Hypoxia, a Novel Inducer of Acute Phase Gene Expression in a Human Hepatoma Cell Line*

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Transcriptional regulation of gene expression by hypoxia is an important, but yet only marginally characterized mechanism by which organisms adapt to low oxygen concentrations. The human hepatoma cell line HepG2 is a widely used model for studying hypoxic induction of the hematopoietic growth factor erythropoietin. In an attempt to identify additional genes expressed in HepG2 cells during hypoxia, we differentially screened a cDNA library derived from hypoxic (1% O₂) HepG2 cells using probes isolated from either normoxic (21% O₂) or hypoxic cells. Two genes were identified, one encoding aldolase, a member of the glycolytic enzymes, and the other encoding α_1 -antitrypsin which belongs to the family of the acute phase (AP) responsive proteins. Whereas hypoxic induction of glycolytic enzymes is well established, oxygen-dependent regulation of AP genes has not been reported so far. AP proteins are liver-derived plasma proteins whose production during inflammation is either up-regulated (positive AP reactants) or down-regulated (negative AP reactants). In the present study, we demonstrate that on the mRNA level hypoxic stimulation of HepG2 cells led to (i) an induction of the positive AP reactants α_1 -antitrypsin, α_1 -antichymotrypsin, complement C3, haptoglobin, and α_1 -acid glycoprotein; (ii) a down-regulation of the negative AP reactant albumin; (iii) an up-regulation of the negative AP reactant transferrin; and (iv) unchanged levels of the positive AP reactants α - and β -fibrinogen as well as hemopexin. Cycloheximide inhibited hypoxic up-regulation of AP mRNAs demonstrating that *de novo* protein synthesis is required for hypoxic induction. Nuclear run-on assays indicate that the hypoxic increase in AP mRNAs is mainly due to transcriptional regulation. The hypoxic response was compared to AP stimulation by interleukin 6. The results suggest that the adaptive response to hypoxia overlaps with, but is not identical with, the AP response mediated by interleukin 6.

Many insights into the mechanisms of oxygen-regulated gene expression have been provided by the study of hypoxia-induced erythropoietin (EPO)¹ gene expression (1–3). The glycoprotein hormone EPO is the predominant stimulator of

erythropoiesis in bone marrow (4, 5). EPO is mainly produced in fetal liver and adult kidney and, to some extent, also in adult liver. Following exposure to hypoxia caused by high altitude or anemia, for example, EPO levels in the blood increase 500- to 2000-fold (6). So far, the human hepatoma cell lines HepG2 and Hep3B are the only permanent cell culture models available to study oxygen-regulated EPO production (7). When cultured under hypoxic conditions (1% versus 21% O₂), both cell lines show an increased EPO secretion which is mainly transcriptionally regulated (8, 9).

Other genes have been found to be induced by hypoxia in many different tissues as well (reviewed in Ref. 10). The wide variety of these genes can be divided roughly into three classes. The first class includes molecules that are favorable for the adaption of the whole organism to general hypoxia, such as EPO which elevates the oxygen transport capacity of the blood. The second class comprises local acting factors that ensure the survival of tissues exposed to local hypoxia due to high oxygen consumption, reduced blood supply, or injury, for example. One example is vascular endothelial growth factor (VEGF), a potent angiogenic factor leading to increased vascularization of the affected tissue. Hypoxic induction of VEGF has been found in many different tissues and tumors (11), as well as in hepatoma cells (12). The third class consists of intracellular factors involved in the adaption of the cell to hypoxia, such as ubiquitously expressed glycolytic enzymes which provide ATP through anaerobic glycolysis (13) or transcription factors of the Jun and Fos family which are induced by low oxygen in cardiac myocytes and hepatoma cells (12, 14). Apart from the assumption that the oxygen sensor might be a hemeprotein (15), neither the nature of this molecule nor the mechanisms leading to enhanced gene expression have so far been characterized clearly (16).

The HepG2 and Hep3B cell lines are not only used extensively to study the regulation of EPO gene expression, but represent also a common model system for investigating proinflammatory, cytokine-dependent expression of acute phase (AP) proteins. The AP response is a protective physiological reaction of the organism to disturbances of its homeostasis due to inflammation caused by tissue injury, infection, or neoplastic growth (reviewed in Refs. 17–19). Characteristics of an AP response after local injury include the release of cytokines (e.g. IL-1, IL-6, IL-11, tumor necrosis factor α , leukemia inhibitory factor, and oncostatin M) which in turn induce a systemic reaction manifested by, for example, fever, elevated secretion of glucocorticoids, and changes in the concentration of a specific set of plasma proteins, termed AP proteins, which are mainly produced in the liver. These AP proteins are either up-regulated (positive AP reactants) or down-regulated (negative AP reactants) during the AP response. Protease inhibitors, blood coagulation factors, transport proteins, and complement components are examples of positive AP reactants which are commonly up-regulated 2- to 10-fold on both the mRNA and protein

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¹ The abbreviations used are: EPO, erythropoietin; AP, acute phase; VEGF, vascular endothelial growth factor; IL, interleukin.

levels. Typical negative AP reactants include albumin and transferrin. IL-6 has been shown to be the major mediator of the AP response in both hepatoma cell lines *in vitro* and in rats *in vivo*, but IL-1, tumor necrosis factor α , leukemia inhibitory factor, and other cytokines are also capable of partially mediating the AP response. The spectrum of AP proteins induced in hepatoma cells, however, varies qualitatively and quantitatively between the different cytokines studied.

Unexpectedly, differential screening of a cDNA library derived from hypoxic HepG2 cells identified hypoxic up-regulation of an AP protein family member, encouraging us to analyze the response of other AP proteins to hypoxia in HepG2 cells.

MATERIALS AND METHODS

Cell Culture—The human hepatoma cell line HepG2, obtained from American Type Culture Collection (ATCC, HB-8065), was cultured in Dulbecco's modified Eagle's medium (high glucose, Life Technologies, Inc.) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (Boehringer Mannheim), 100 units/ml penicillin, 100 μ g/ml streptomycin, non-essential amino acids, 2 mM L-glutamine, and 1 mM sodium pyruvate (all Life Technologies, Inc.) in a humidified atmosphere at 37 °C and 5% CO₂. Oxygen tensions (pO₂) in the incubator (Forma Scientific) were either 140 mm Hg (21% v/v, normoxia) or 7 mm Hg (1% v/v, hypoxia). For hypoxic induction, subconfluent HepG2 cells were trypsinized, diluted in 100 μ l/cm² medium, and allowed to recover for 24 h. The medium was replaced, and incubation at 1% O₂ was started. Cell density at this point was 1×10^4 /cm². For translation inhibition, 20 μ g/ml cycloheximide (Sigma) was added, and, for cytokine induction, the media contained 20 ng/ml IL-6 (R & D Systems) and 1 μ M dexamethasone (Serva).

Library Construction and Differential Screening—HepG2 cells were induced at 1% O₂ for 16 h, and total RNA was isolated using the acid guanidine thiocyanate-phenol-chloroform method (20). In order to minimize the effects of reoxygenation, care was taken to immediately lyse the cells after removing them from the hypoxic incubator. Polyadenylated mRNA was obtained by two rounds of oligo(dT)-cellulose spin column chromatography (Pharmacia Biotech Inc.). A phage λ -cDNA library was constructed using the Uni-ZAP XR vector according to the manufacturer's instructions (Stratagene). Replica filters from this library were differentially screened with ³²P-labeled total cDNA from either hypoxic or normoxic HepG2 cells. cDNA synthesis was performed for 1 h at 41.5 °C in 25 μ l of 50 mM Tris/Cl (pH 8.3), 60 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 1 unit/ μ l RNasin (Promega), 1 mM each dATP, dGTP, dTTP, 5 μ M dCTP, using 2 μ g of denatured (70 °C, 4 min) poly(A)⁺ mRNA, 2 μ g of oligo(dT)₁₂₋₁₈ (Pharmacia), 50 μ Ci of [α -³²P]dCTP (Amersham), and 400 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The RNA strand was then removed by alkaline hydrolysis (0.5 M NaOH) for 15 min at 55 °C, and the cDNA was purified by Sephadex G-50 (Pharmacia) chromatography as described (21). Hybridization to the filters was performed using standard protocols (21). Clones which showed a stronger signal with the hypoxic cDNA probe compared to the normoxic probe were picked, plaque-purified, and *in vivo*-excised using the Ex-Assist helper phage system (Stratagene). Single-stranded DNA was prepared (21) to sequence the clones with the dideoxy chain termination method (22). The clones were identified by comparing their sequence to the GenBank™/EMBL data library using the GCG program package (23).

EPO Determination—EPO protein concentrations in the cell culture supernatants were determined by a radioimmunoassay using ¹²⁵I-EPO (Amersham) and recombinant human EPO (Boehringer Mannheim) as described previously (24).

Quantitative Northern Blot Analysis—Total RNA (10 μ g) isolated from HepG2 cells was denatured in formamide/formaldehyde and electrophoresed through a 1% agarose gel containing 6% formaldehyde as described (21). Following pressure blotting (Stratagene) to nylon membranes (Biodyne A, Pall) and UV-cross-linking (Stratalinker, Stratagene), the filters were hybridized to labeled DNA probes in 50% formamide, 10% dextran sulfate, 5 \times Denhardt's solution, 200 μ g/ml sonicated salmon sperm DNA, 1% SDS, 0.9 M NaCl, 60 mM NaH₂PO₄, 6 mM EDTA (pH 7.0) for 14 h at 42 °C. The filters were washed to a final stringency of 55 °C in 0.1 \times SSC, 0.2% SDS and either exposed to x-ray films using an intensifying screen (QUANTA III, DuPont NEN) or quantified using a PhosphorImager (Molecular Dynamics). DNA probes were obtained by restriction digestion of the plasmid followed by agarose gel purification and labeled to a specific radioactivity of 1×10^9

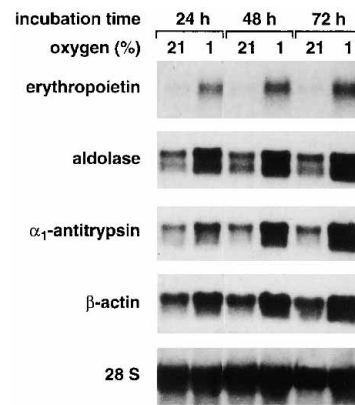


FIG. 1. Northern blot analysis of hypoxia-induced HepG2 cells. HepG2 cells were induced by exposure to 1% O₂ for 24 h to 72 h at an initial cell density of 1×10^4 /cm². Normoxic (21% O₂) control experiments were performed in parallel for each time point. Equal amounts (10 μ g) of total RNA were loaded. The signal obtained with a 28 S rRNA probe was used to control for equal loading and blotting efficiency.

dpm/ μ g (Prime-It II, Stratagene). The following plasmids were purchased from the source noted: α ₁-antichymotrypsin, pHACT235 (ATCC 61600); complement C3, pHLC3 (ATCC 59108); transferrin, pTf (ATCC 57228); albumin, pILMALB5 (ATCC 61356); α -fibrinogen, p304 (ATCC 59706); β -actin (Clontech). The following plasmids were kindly provided by the individuals noted: haptoglobin, pHp/6, α ₁-acid glycoprotein, p8AGP, and hemopexin, pHpxII (V. Poli); β -fibrinogen (P. C. Heinrich); rat ribosomal RNA, p19 (I. Stancheva); and erythropoietin, pe49f (C. Shoemaker, Genetics Institute). The VEGF plasmid pmVh was obtained by subcloning a reverse transcription-polymerase chain reaction amplification product of mouse brain RNA using the primers 5'-cggaatcGCGGGCTGCCTCGCAGTC-3' (sense) and 5'-cggaatcTCACCGCCTTGGCTTGTCAC-3' (antisense) which span the entire coding region (25). The ribosomal protein L28 cDNA was cloned from the HepG2 λ phage library.² Unless otherwise stated, all cDNAs were of human origin.

Determination of Transcription Rates—Nuclear run-on assays were performed as described previously (26). Briefly, nuclei from 5×10^7 HepG2 cells cultured at normoxia or hypoxia for 48 h were isolated, and transcriptional run-on reactions were performed in the presence of 250 μ Ci of [α -³²P]UTP. The purified reaction products were hybridized to dot-blot filters containing each 5 μ g of a linearized plasmid DNA. Following RNase A and proteinase K treatment, the signal intensities were evaluated by PhosphorImager analysis. Northern blot analysis of parallel cultures was performed to determine the steady-state mRNA levels as described above.

RESULTS

Differential Screening of a HepG2 cDNA Library Reveals Hypoxic Induction of Aldolase and α ₁-Antitrypsin mRNA—In an attempt to clone new, hypoxia-regulated genes, we constructed and differentially screened a phage λ -cDNA library derived from HepG2 cells cultured at 1% O₂ for 16 h. These moderate hypoxic conditions did not significantly influence cell growth and morphology compared to the normoxic control. Following low density plating of 50,000 λ clones, 8 clones were selected that hybridized more strongly to total cDNA probes derived from hypoxic HepG2 cells compared with total cDNA derived from normoxic cells. Northern blot analysis revealed that 2 out of these 8 clones corresponded to hypoxia-inducible genes.

The first clone was identical with fructose-1,6-bisphosphate aldolase A, starting 18 base pairs upstream of the AUG translation initiation codon (27). Northern blot hybridizations (Fig. 1) revealed a time-independent 2- to 3-fold accumulation of aldolase mRNA during 24 to 72 h of hypoxia in HepG2 cells (see below). This result was consistent with nuclear run-off experiments in skeletal muscle cells, where aldolase transcription

² C. Schuerer-Maly and R. H. Wenger, unpublished results.

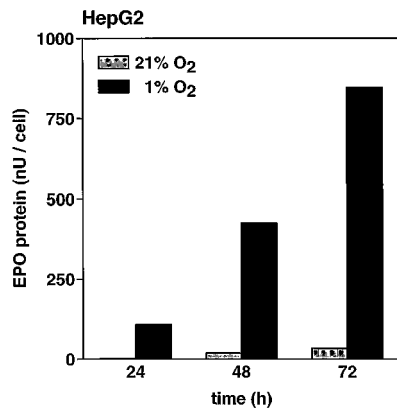


FIG. 2. EPO protein levels in the supernatant of HepG2 cultures. HepG2 cells were induced by hypoxia as described in Fig. 1. EPO concentrations were determined by a radioimmunoassay using recombinant human EPO. Results are expressed as nanounits of EPO per cell.

rates have been reported to be induced 2- to 5-fold by low oxygenation (13). During hypoxia, when anaerobic glycolysis is the major source of ATP, the induction of glycolytic activity ensures constant energy supply to the cell (28). The cloning of a hypoxia-inducible glycolytic enzyme, however, confirmed the accuracy of our differential screening approach.

The second clone was identified as α_1 -antitrypsin, beginning 11 bp upstream of the translational start site (29, 30). Its mRNA was found to be induced in a time-dependent manner in HepG2 cells (Fig. 1). Since α_1 -antitrypsin belongs to the group of plasma proteins induced in hepatocytes during the AP response (17), we tested if other members of this family are induced by hypoxia as well.

Hypoxia Modulates Expression of mRNAs Encoding AP Proteins in HepG2 Cells—A time course of hypoxic induction of HepG2 cells was performed, including a normoxic control for every time point. Because inducibility of EPO expression in HepG2 cells has previously been shown to be reciprocally dependent on cell confluency (7, 31), the cells were induced at a low cell density for 24 to 72 h (see “Materials and Methods”). After 72 h of hypoxic induction, experiments were terminated since hypoxic passaging of cells was not practicable. Cell viability after hypoxic incubation remained unchanged as judged by trypan blue exclusion. In order to monitor the extent of hypoxic induction of HepG2 cells, the EPO concentration in the conditioned medium of every time point was determined by radioimmunoassay. As shown in Fig. 2, EPO levels increased under hypoxic conditions to 107 nanounits/cell after 24 h and 846 nanounits/cell after 72 h, corresponding to a maximum accumulation of 25-fold after 72 h of hypoxia. This induction was much higher than the 2- to 3-fold induction published previously for this cell line (7), most probably due to optimized cell culture conditions.³ When cells were plated at 8-fold higher density, the inducibility decreased even though confluency was still not reached (data not shown).

Northern blot analysis was then performed using hybridization probes for EPO and VEGF, as well as positive AP reactants (α_1 -antitrypsin, α_1 -antichymotrypsin, complement C3, haptoglobin, α_1 -acid glycoprotein, hemopexin, and α - and β -fibrinogen) and negative AP reactants (albumin and transferrin). The signals were quantitated by PhosphorImager analysis and corrected for differences in loading and blotting by hybridization to a 28 S ribosomal probe as exemplified in Fig. 1. β -Actin, another commonly used normalization probe, was found to be reproducibly induced by a factor of approximately 1.5 (Figs. 1

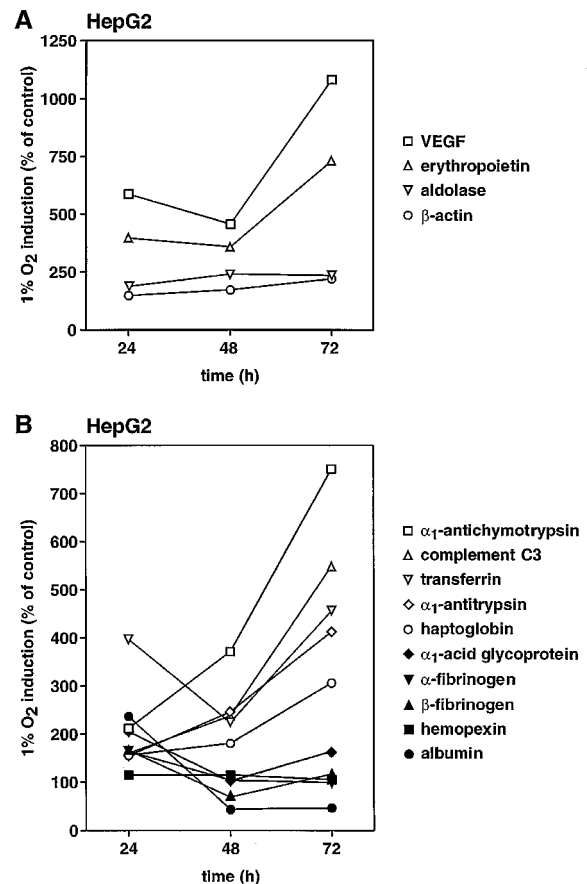


FIG. 3. Time-dependent induction of steady-state mRNA levels following hypoxic treatment of HepG2 cells. Northern blots were quantified by PhosphorImager analysis and normalized by hybridization to a 28 S rRNA probe. Results are shown as percentage of the corresponding normoxic control. A, hypoxic induction of non-AP genes. B, hypoxic induction of AP genes.

and 3A). A weak transcriptional activation of β -actin by hypoxia has already been reported in rat skeletal muscle cells (13). Hence, β -actin is inappropriate for normalization of these experiments. Hypoxic response of HepG2 cells was verified by analyzing the induction rates for EPO and VEGF. Under the stated experimental conditions, EPO and VEGF mRNAs were induced 3.5- to 7-fold and 4.5- to 11-fold, respectively, after 24 h to 72 h of hypoxia (Fig. 3A). Fig. 3B revealed a similar 3- to 7-fold time-dependent induction of AP protein-encoding mRNAs (α_1 -antichymotrypsin, complement C3, α_1 -antitrypsin, and haptoglobin) after 48 h to 72 h of hypoxic incubation. The negative AP reactant albumin was down-regulated by hypoxia, as it is during the *in vivo* AP response. Surprisingly, although fibrinogens are among the most prominent positive AP reactants induced by IL-6 in HepG2 cells (17), mRNA levels of the coordinately expressed α - and β -fibrinogen genes (32, 33) were not significantly affected by hypoxia. The positive AP reactants α_1 -acid glycoprotein and hemopexin were also only marginally regulated by oxygen. Moreover, transferrin, which is down-regulated by IL-6 and tumor necrosis factor α in HepG2 cells *in vitro* and also during the AP response *in vivo* (17), was induced by hypoxia up to 4.5-fold. Hypoxic exposure for up to 24 h at an 8-fold higher initial cell density did not significantly induce mRNA levels of AP proteins or of EPO or VEGF (not shown), in accordance with the previously reported cell density dependence of EPO expression in HepG2 cells (7). Time course and extent of hypoxic induction of AP mRNAs was similar to the mRNAs encoding EPO and VEGF. A maximum was reached at

³ R. H. Wenger, unpublished observations.

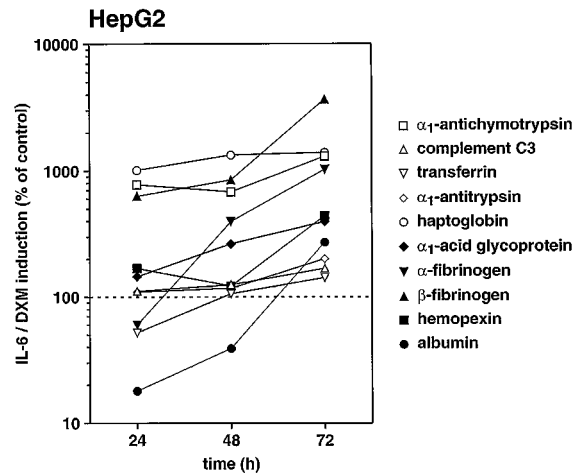


FIG. 4. **IL-6 induction of AP genes in HepG2 cells.** The cells were induced with 20 ng/ml IL-6 in the presence of 1 μ M dexamethasone (DXM). Steady-state mRNA levels were determined as described in Fig. 3.

72 h of incubation, whereas hypoxic induction of aldolase mRNA remained relatively constant over the time points examined (Fig. 3A). In summary, these results suggest a common mechanism for hypoxic induction of EPO, VEGF, and AP mRNAs and possibly a distinct mechanism for induction of the glycolytic enzyme aldolase.

Hypoxia Regulates a Specific Set of AP Genes in HepG2 Cells—Among the proinflammatory cytokines, IL-6 has been reported to be the major activator of AP gene expression in hepatoma cells (17). To compare the hypoxic AP induction with cytokine stimulation, HepG2 cells were cultured up to 3 days in the presence of 20 ng/ml IL-6 and 1 μ M dexamethasone, which is known to be required for full IL-6 activation (17). The results of the combined IL-6/dexamethasone treatment of HepG2 cells, performed under the same experimental conditions as for hypoxic stimulation, are shown in Fig. 4. A strong induction (7- to 35-fold) was observed for haptoglobin, α_1 -antichymotrypsin, and the fibrinogen genes. α_1 -Acid glycoprotein, hemopexin, complement C3, and α_1 -antitrypsin were only moderately induced (1.5- to 4-fold). Albumin and transferrin were down-regulated by IL-6/dexamethasone after 1 to 2 days. However, this effect could be reversed after long-term (3 days) treatment. Dexamethasone alone did not influence AP gene expression either under normoxic or hypoxic conditions, and the combination of IL-6/dexamethasone with hypoxia did not result in any additive effects (not shown).

A comparison of hypoxic and IL-6/dexamethasone treatment of HepG2 cells *in vitro* opposed to the AP response *in vivo* is shown in Table I. The results suggest that most of the liver AP genes can also be induced by IL-6/dexamethasone in HepG2 cells. However, Table I revealed a hypoxia-specific AP mRNA pattern in HepG2 cells which was overlapping but not identical with the cytokine-induced pattern observed *in vitro* and *in vivo*. This pattern also did not correspond to the two classes of AP proteins, proposed by Baumann and Gaudie (34) based on their hormone requirement.

Transcriptional Regulation of Hypoxic AP Gene Expression—To determine to what extent transcriptional and post-transcriptional mechanisms contribute to the hypoxic increase of AP steady-state mRNA levels, Northern blot and nuclear run-on assays were performed using HepG2 cells which were induced for 48 h. Following background subtraction of the signal obtained with the vector alone, the induction rates were normalized to the signal derived from the ribosomal protein L28 cDNA probe. Normalization to L28 was chosen because, in

TABLE I
Regulation of AP mRNAs by hypoxia and IL-6/dexamethasone in HepG2 cells *in vitro* and AP response in human serum *in vivo*

AP protein	HepG2 cells ^a		Human serum ^a
	1% O ₂	IL-6/dexamethasone	AP response ^b
α_1 -Antichymotrypsin	+	+	+
Complement C3	+	(+)	+
Transferrin	+	— ^c	—
α_1 -Antitrypsin	+	(+)	+
Haptoglobin	+	+	+
α_1 -Acid glycoprotein	(+)	+	+
α -Fibrinogen	0	+	+
β -Fibrinogen	0	+	+
Hemopexin	0	+	0
Albumin	—	— ^c	—

^a 0, no effect; —, inhibition; (+), ≤ 2 -fold induction; +, induction.

^b Ref. 17.

^c Weak IL-6 induction after prolonged incubation.

contrast to the glycolytic enzymes and β -actin (see above), L28 was not regulated by oxygen concentrations.² The induction rate in this particular set of experiments was rather low. However, the rates were comparable with those obtained with VEGF and aldolase probes, suggesting that the overall stimulation of the cells was somewhat reduced. The comparison of the induction rates in Northern blots and run-on assays shown in Fig. 5 indicates that hypoxic induction of the AP mRNAs encoding α_1 -antichymotrypsin, transferrin, and α_1 -antitrypsin is due mainly to the change in transcription rates.

Hypoxic Induction of AP mRNA Levels Is Translation-dependent—Cycloheximide, a potent inhibitor of translation, has been reported to block the hypoxic induction of mRNAs encoding EPO (15) and glycolytic enzymes (35, 36), most probably by inhibiting the synthesis of a transcription factor required for oxygen-regulated EPO gene expression (37). To test whether AP genes are induced by hypoxia through a similar mechanism, we exposed HepG2 cells to 1% O₂ for 48 h with or without 20 μ g/ml cycloheximide. As shown in Fig. 6, this treatment abrogated hypoxic induction of α_1 -antichymotrypsin, transferrin, and α_1 -antitrypsin mRNAs, indicating that a *de novo* translated protein is required for hypoxic stimulation, as it is known for the induction of EPO and glycolytic enzymes.

DISCUSSION

Putative Physiological Functions of Hypoxic AP Protein Induction—The results presented in this study are the first demonstration of hypoxic induction of AP genes in HepG2 cells, a hepatoma cell line known to express EPO (7) and VEGF (this study) in an oxygen-dependent manner. Assuming that hypoxic modulation of AP genes in cell culture reflects the *in vivo* situation, this finding might have important implications for the mechanisms through which an organism responds to low oxygen supply. The widespread oxygen-sensing and signaling mechanisms leading to increased expression of specific genes such as EPO might affect other genes also, including AP responsive genes, whose products could play a role for the adaptation of the organism to hypoxic conditions. It is attractive to postulate that the AP proteins are involved in maintaining efficient oxygen uptake and enhancing oxygen transport capacity.

Both, α_1 -antitrypsin and α_1 -antichymotrypsin inhibit proteases (neutrophil-derived elastase and cathepsin G, respectively) that otherwise could lead to proteolytic degradation of lung tissue and emphysema, thereby affecting general oxygen supply to the organism (38, 39). It is tempting to speculate that induction of these protease inhibitors might contribute to protecting the lung from tissue damage caused by neutrophils which are known to invade the lung of hypoxic mice (40).

Since iron is an essential component for hemoglobin synthe-

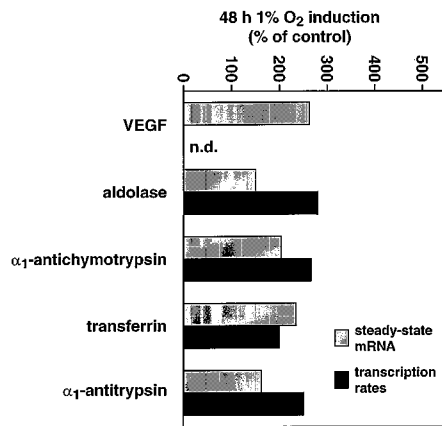


FIG. 5. Transcriptional regulation of hypoxia-inducible AP gene expression. Northern blot analysis and nuclear run-on assays were performed using parallel HepG2 cultures induced at 1% O₂ for 48 h. Signal intensities were corrected for vector background, normalized to the signal obtained with a ribosomal protein L28 cDNA probe, and expressed as percentage of the normoxic control. The mean values of two independent experiments are shown. Run-on signals for VEGF were less than 2-fold above background level and hence not included in this figure. *n.d.*, not detectable.

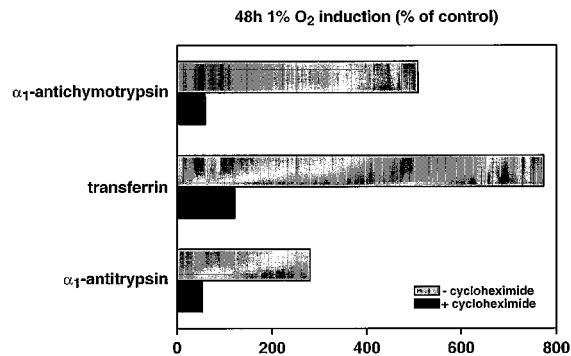


FIG. 6. Effect of cycloheximide on hypoxic AP gene induction. HepG2 cells were induced for 48 h at 1% O₂ with or without 20 μg/ml cycloheximide, and total RNA was analyzed by Northern blotting as described in Fig. 3.

sis during red cell formation in bone marrow, hypoxic up-regulation of the iron transport protein transferrin might support EPO-induced erythropoiesis by enhanced iron supply. Indeed, increased transferrin serum levels in mice (41) and rats (42) that were exposed to 50% atmospheric pressure for 1 to 3 days have been reported. Likewise, hypoxic induction of the hemoglobin-binding transport protein haptoglobin might sustain enhanced erythropoiesis by preventing the loss of heme iron from the kidney. Besides many other functions, complement C3 is involved in erythrocyte degradation, thereby maintaining constant erythrocyte lifespan (reviewed in Refs. 43 and 44). Complement C3 induction might be required to keep the balance between plasma complement C3 concentrations and decay accelerating factor under conditions of enhanced erythropoiesis. Decay accelerating factor is present on the surface of erythrocytes and protects them from complement-mediated cell lysis.

Putative Pathological Functions of Hypoxic AP Protein Induction—Hypoxia-induced AP gene expression might also confer pathological processes. In Alzheimer's disease, for example, increased expression of α₁-antichymotrypsin and complement components such as C3 have been reported (45, 46). A tight association between α₁-antichymotrypsin and βA4-protein in senile β-amyloid plaques suggests that α₁-antichymotrypsin might be involved in the pathophysiology of this disease. In-

deed, α₁-antichymotrypsin has been demonstrated to promote assembly of Alzheimer β-protein into filaments *in vitro* (47). A brain-specific AP response has been proposed because astrocytes, which synthesize α₁-antichymotrypsin and complement C3, respond to brain-derived cytokines (48–50). However, a cause for increased cytokine expression in the brain has not clearly been established (51). Based on the results presented in this study, we postulate that hypoxia could stimulate α₁-antichymotrypsin and complement C3 production. In support of the postulate that the brain can produce AP proteins in an oxygen-dependent manner, we recently found hypoxia-inducible EPO expression in brain (52). Further experiments suggest that astrocytes are the site of brain EPO expression (53).⁴ Hypoxic stimulation of primary astrocytes will be performed to investigate if α₁-antichymotrypsin is also inducible by low oxygen concentrations in this cell type. Hypoxic induction of α₁-antichymotrypsin and other AP proteins in astrocytes might provide new insights into the molecular pathogenesis of plaque formation in the brain.

Is There a Common Mediator of Oxygen- and Cytokine-dependent AP Gene Induction?—Our finding that most AP responsive genes are inducible in an oxygen-dependent manner raises the question as to whether every AP gene is individually regulated by either hypoxia or proinflammatory cytokines or whether a common oxygen- and cytokine-dependent factor is able to regulate AP gene expression. Possible common mechanisms might include HepG2-derived hypoxia-inducible cytokines, protein kinases, and/or transcription factors. Interestingly, IL-6 has recently been shown to be hypoxia-inducible in astrocytes (54) and endothelial cells (55). The IL-6 activating transcription factor C/EBPβ seems to be critically involved in hypoxic IL-6 induction (55). Thus, hypoxia-inducible IL-6 might be a candidate mediator of AP gene induction in HepG2 cells. However, the question of whether parenchymal hepatocytes are able to produce IL-6, which in turn could activate hepatic gene expression in an autocrine fashion, is a matter of debate (56, 57). In addition, the pattern of genes induced by either IL-6 or hypoxia (see Table I) is clearly different, providing evidence that hypoxic AP gene expression cannot simply be attributed to an autocrine IL-6 activation. Since the transcription factor C/EBPβ is not only involved in IL-6 induction observed in nonhepatic cells, but also mediates IL-6-induced AP gene expression in hepatocytes (58), we are currently examining the effect of hypoxia on the C/EBP family of transcription factors.

Cellular stress could be another common inducer of AP proteins and EPO under hypoxic conditions. Heat shock is a well-established activator of stress-responsive genes and the question arose whether heat shock and hypoxia are two different stimuli resulting in the expression of the same genes. Several lines of evidence indicate that this is not the case. Despite reports that anoxia (59) and anoxia followed by reoxygenation (60) induce heat-shock proteins in mammalian cell lines, oxygen tensions similar to those used in our experiments failed to induce the major heat-shock proteins (3). On the other hand, IL-6 does not induce the heat-shock protein Hsp70 in Hep3B cells (61). Furthermore, neither EPO (3, 15) nor AP proteins (60) are induced by heat shock in hepatoma cells. In this context, it is noteworthy that although the oxygen tension in our experiments (7 mm Hg) is 21-fold reduced compared to ambient air (140 mm Hg), it represents only about a 3.5-fold reduction compared to the average oxygen tension measured *in vivo* at the liver surface (approximately 25 mm Hg, Ref. 62). This

⁴ H. H. Marti, R. H. Wenger, L. A. Rivas, U. Stranmann, M. Digicaylioglu, V. Henn, Y. Yonekawa, C. Bauer, and M. Gassman, submitted for publication.

suggests that the hypoxic conditions used in our experiments represent a rather mild physiological stimulant compared to the pathological conditions of anoxia or heat shock, both of which result in heat-shock protein expression.

In summary, inflammation-independent hypoxic induction of AP protein gene expression in hepatic and probably also extra-hepatic tissues could shed new light on the mechanisms by which organisms adapt to hypoxia.

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